

# THE ESTABLISHMENT OF A NEW OLIGOTRICH GENUS *VARISTROMBIDIUM* GEN. NOV. AND THE MORPHOLOGY AND PHYLOGENY OF A MARINE CILIATE, *VARISTROMBIDIUM KIELUM* (MAEDA AND CAREY, 1985) NOV. COMB. (PROTISTA, CILIOPHORA)

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**Abstract** A Chinese population of the oligotrich ciliate *Strombidium kielum* Maeda & Carey, 1985 was discovered in sand on the coast of Qingdao, China, and an analysis of morphological data from live cells and protargol preparations allowed this poorly described species to be redefined. A unique, heretofore undescribed pattern of somatic ciliation was discovered that revealed this species to be separated from other taxa in its family at the generic level. Therefore, it was assigned to the new genus *Varistrombidium*, which is characterized by possession of 5 somatic kineties that run obliquely across the ventral side of the cell and having somatic kineties 1 and 2 extending to the dorsal side and ending at the caudal area. Also, the secondary structure of variable region 2 of SSrRNA gene in *V. kielum* was predicted and compared with its morphologically similar congeners. Furthermore, some supplementary data of *Apostrombidium pseudokielum* Xu *et al.*, 2009, a newly reported organism, is also provided.

**Key words** SSrRNA, secondary structure, Yellow Sea, China.

## 1 Introduction

Faunistic surveys of the biodiversity of ciliated protozoa in marine habitats in the region of Qingdao, on the coast of the Yellow Sea in Northeastern China, has resulted in reports of many new or little-known oligotrich ciliates (Xu and Song, 2006; Xu *et al.*, 2006; Xu *et al.*, 2009). During these studies, we discovered a ciliate which was identified as *Strombidium kielum*. The somatic ciliature of this ciliate, which is revealed by protargol staining, is unique among oligotrich ciliates. Thus we assign it to a new genus *Varistrombidium*. Here we present a morphological redescription of this species and an analysis of its phylogenetic position using morphological characters and sequences of the gene coding for SSrRNA.

Many studies have been carried out among different eukaryotic taxa to construct phylogenies inferred from the information of ITS (internal transcribed spacer regions) secondary structures (Felinor and Rosselló, 2007; Liu and Schardl, 1994). However, knowledge of secondary structure in ciliates is still very rare, comprising only several previous studies (Coleman, 2005; Sun *et al.*, 2010). In present study, we focused on the secondary structure of *Varistrombidium kielum* and its 16 morphologically similar species which belong to 8

different genera. This is the first time that secondary structures have been used in phylogenetic analysis of oligotrich ciliates. Finally, some supplementary data of *Apostrombidium pseudokielum* Xu *et al.*, 2009, a newly reported organism, is also provided since the original description lacks some detailed information.

## 2 Materials and Methods

### 2.1 Collection of samples and observation of morphology

Ciliates were sampled from coastal sands at a depth of 20 cm in the Yellow Sea off Qingdao (36°08'N, 120°43'E) on 15 Mar. 2006. Cells were isolated from sediment and maintained in the laboratory for several days at room temperature (ca. 22 °C) for further study. Protargol impregnation was done according to the protocol of Wilbert (1975) to reveal the infraciliature. Specimens were observed in vivo with phase contrast and differential interference contrast microscopy. Counts, drawings (with help of a Camera Lucida) and measurements were performed at a magnification of  $\times 1\,250$ .

### 2.2 DNA extraction, PCR amplifying and sequencing

DNA was isolated according to Chen and Song (2001). Universal oligonucleotide primers (Medlin

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*et al.*, 1988) were used to amplify the SSrRNA gene. Purified PCR product of appropriate size was inserted into the pUCm-T vector (Shanghai Sangon Biological Engineering & Technical Service Company) and sequenced at the Invitrogen sequencing facility in Shanghai, China.

### 2.3 Phylogenetic analyses and construction of secondary structures

All available SSrRNA gene sequences of oligotrich ciliates from Genbank were included in present analyses and their accession numbers were shown in Fig. 35. The Maximum Parsimony (MP) and Neighbor-Joining (NJ) analyses were performed with PAUP\* 4.0b10 (Swofford, 2002), and the support for the internal branches was estimated using the bootstrap method with 1 000 replicates (Zhang *et al.*, 2010).

Secondary structures of the variable regions of SSrRNA sequences in all oligotrich ciliates were determined by submission of primary sequences to the RNA-folding website supporting MFOLD version 3.2 (Zuker *et al.*, 1999).

## 3 Results

### Family Strombidiidae Fauré-Fremiet, 1970

#### Genus *Varistrombidium* gen. nov.

##### 3.1 Diagnosis

Strombidiidae with 5 spirally arranged somatic kineties which run obliquely across ventral side and parallel to each other, with the longest 1 and 2 extending to dorsal side and terminating at caudal area.

Type species. *Varistrombidium kielum* (Maeda & Carey, 1985) comb. nov. (by monotype).

Type locality. A sandy beach near Qingdao (39°10'N, 117°06'E), China.

Deposition of type material. Two permanent voucher slides of protargol impregnated specimens are deposited in the Natural History Museum, London and the Laboratory of Protozoology, OUC, China with registration Nos. 2005:03:01:01 and 2005:03:01:02, respectively.

Etymology. Combined with Vari- and the known name *Strombidium*, indicating that it differs from the latter; neutral gender.

#### *Varistrombidium kielum* (Maeda and Carey, 1985) nov. comb.

Basionym: *Strombidium kielum* Maeda and Carey, 1985

##### 3.2 Improved diagnosis

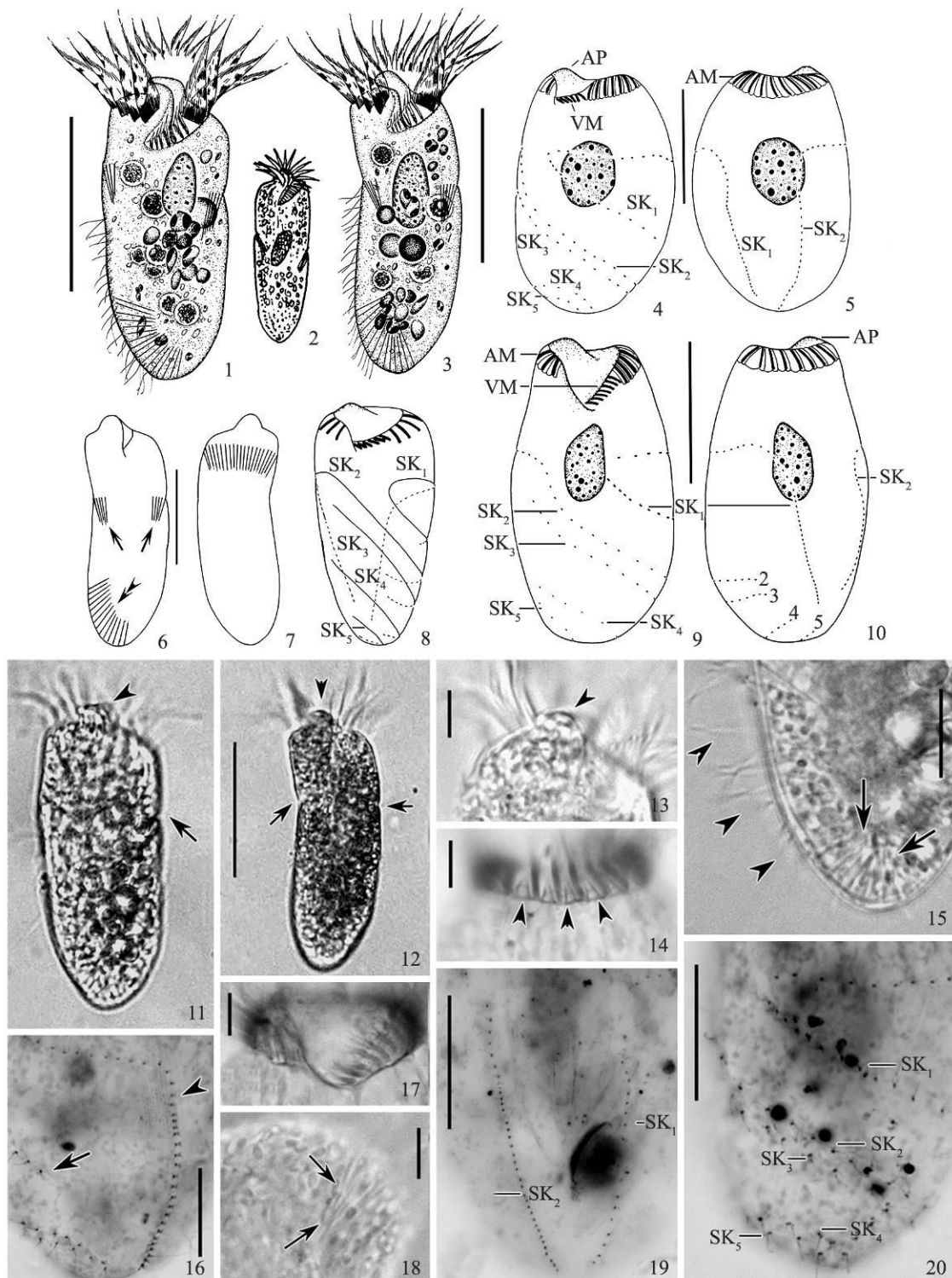
Marine *Varistrombidium* measuring 65 – 85  $\mu\text{m}$   $\times$  25 – 40  $\mu\text{m}$  in vivo; cell uniformly elongate and barrel-shaped, with apical protrusion on right side of anterior end; one ovoid to ellipsoid macronucleus; extrusomes

distributed on dorsal shoulders and ventral side of caudal area; buccal cavity extending for approximately 1/5 of distance toward posterior of cell; 15 – 17 anterior and 7 – 8 ventral membranelles; 5 parallel, obliquely oriented somatic kineties extending across ventral side of cell; somatic kineties 1 and 2 extending to dorsal side and nearly to posterior end before terminating.

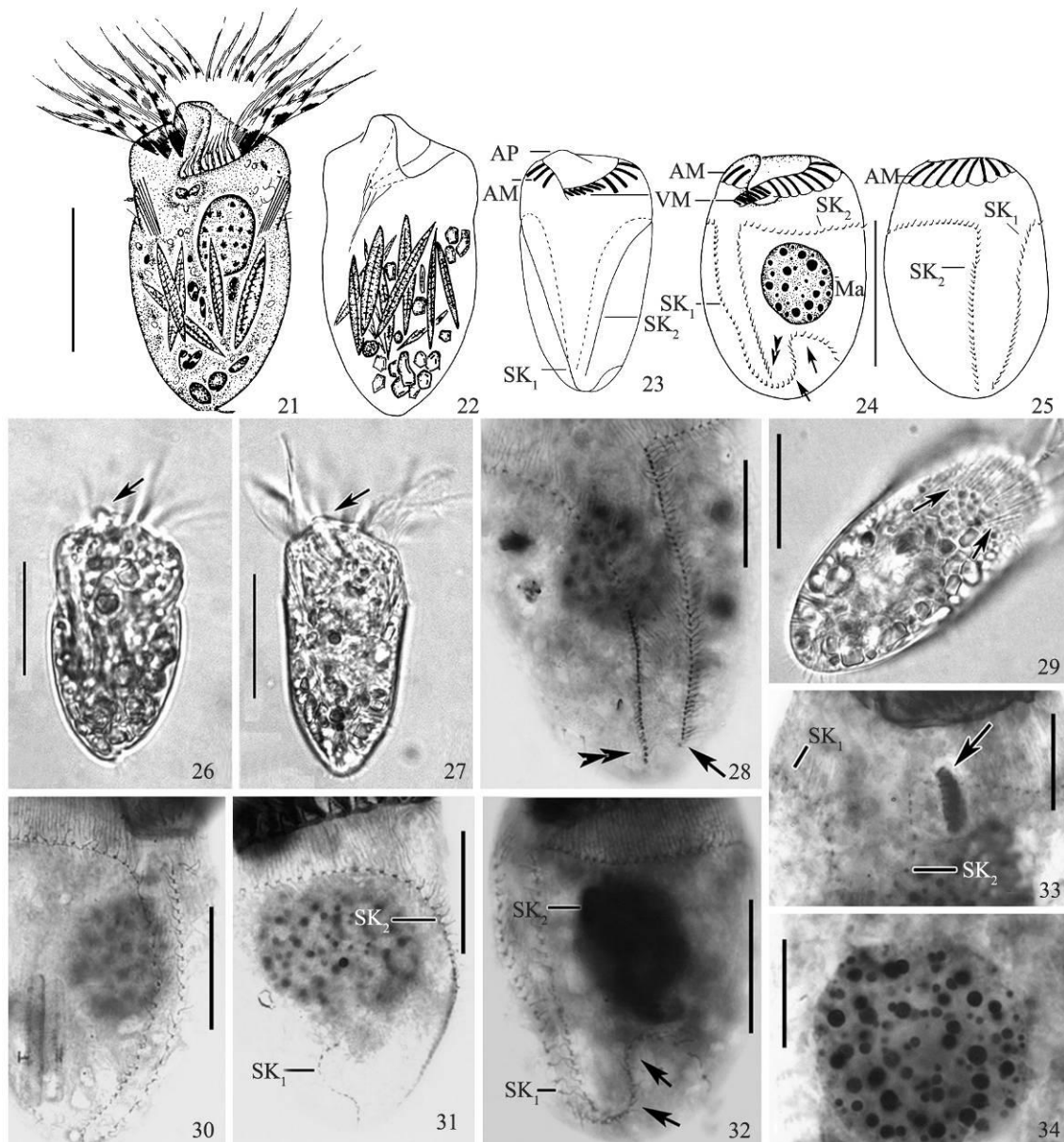
##### 3.3 Morphology of the Qingdao population

Living cells measuring approximately 75  $\mu\text{m}$   $\times$  30  $\mu\text{m}$ , elongate, barrel-shaped, slightly asymmetric, generally constant in form (Figs 1, 3, 11 – 12). Upper equatorial area usually narrowed (Figs 11 – 12, arrows) when viewed from ventral side, posterior end of cell usually bluntly acuminate. Collar with conspicuous apical protrusion on right side; protrusion approximately 5  $\mu\text{m}$  high, sometimes disappearing or becoming indistinct after fixation (Figs 11 – 13, arrowhead). Buccal cavity shallow, terminating approximately 1/6 – 1/5 of distance toward posterior end of cell. Cell fragile, sensitive to presence of coverslip and easily bursting with increase in temperature or contact with surface of water. Hemitheca not detected. Extrusomes prominent, acicular, approximately 10  $\mu\text{m}$  long, not in bundles, evenly distributed; extrusomes found along shoulders and narrowed upper equatorial area of dorsal side and on ventral side of caudal area (Figs 6 – 7, arrows and double arrowheads; Fig. 15, arrows). Cytoplasm transparent, containing food vacuoles filled with diatoms, flagellates, and other prey items whose presence can make cells partly opaque or even dark at lower magnifications (Figs 11 – 12, 15). Macronucleus ovoid to ellipsoid, located in anterior half of cell, containing numerous small, globular nucleoli measuring approximately 2 – 4  $\mu\text{m}$  in diameter (Figs 4 – 5, 9 – 10). Micronucleus, contractile vacuole, and cytophyge not observed.

Oral apparatus occupying entire anterior end of cell. Adoral zone of membranelles with distinct ventral opening; clearly divided into anterior part consisting of 15 – 17 membranelles and ventral part consisting of 7 – 8 membranelles (Figs 4 – 5, 9 – 10, 17). All membranelles composed of polykinetids consisting of 3 basal bodies each, visible in protargol preparations as three rows of basal bodies. Bases of anterior membranelles approximately 6  $\mu\text{m}$  long and bases of ventral membranelles approximately 3 – 4  $\mu\text{m}$ . Cilia in anterior membranelles approximately 25  $\mu\text{m}$  long in vivo, extended toward anterior when swimming (Figs 11 – 12). Endoral membrane not observed either in living or protargol-stained specimens, probably very short. Argentophilic fibers lying between and oriented parallel to anterior membranelles (Fig. 14, arrowheads).



Figs 1–20. *Varistrombidium kielum* from live cells (1–3, 6–7, 11–13, 15, 18) and after protargol impregnation (4–5, 9–10, 14, 16–17, 19–20) (Fig. 2 from Kahl, 1932, called *Strombidium* sp.). 1. Ventral view of a representative specimen. 2. Ventral view of a specimen from the type population collected at Kiel, Germany (from Kahl, 1932). 3. To show different cell shape. 4–5. Left lateral and right dorsal views of the same specimen. 6–7. To show the distribution of extrusomes. 8. Scheme, to demonstrate the arrangement pattern of somatic ciliature. 9–10. Ventral and dorsal views of the same specimen. 11–12. Ventral views of two specimens, to show different cell shapes, arrowhead marks the apical protrusion while arrows indicate the constriction along the upper equatorial region. 13. To show the apical protrusion (arrowhead). 14. Anterior end, arrowheads mark the argentophilic fibres between and parallel to anterior membranelles. 15. Posterior portion, arrowheads show the long somatic cilia in the posterior area of cell, while arrows to show the extrusomes. 16. Dorsal view, arrowhead marks the kinety 1, while arrow indicates the kinety 2. 17. Anterior portion of cell, to show the anterior and ventral membranelles. 18. Arrows indicate the extrusomes on dorsal side. 19. Dorsal view, to show the kinety 1 and 2. 20. Ventral view to show the somatic ciliature. Abbreviations: AM. Anterior membranelles. AP. Apical protrusion. Ma. Macronucleus. SK<sub>1</sub>. Somatic kinety 1. SK<sub>2</sub>. Somatic kinety 2. SK<sub>3</sub>. Somatic kinety 3. SK<sub>4</sub>. Somatic kinety 4. SK<sub>5</sub>. Somatic kinety 5. VM. Ventral membranelles. Scale bars: 1–3, 11–12 = 40  $\mu$ m; 4–7, 9–10, 15–16, 19–20 = 30  $\mu$ m; 13–14, 17–18 = 5  $\mu$ m.



Figs 21 – 34. Morphology and ciliary pattern of *Apostrombidium pseudokiehni*, as viewed from life observation (21 – 23) and after protargol staining (24 – 25). 21. Ventral view of a typical specimen. 22. To show the ingested algae. 23. To demonstrate the arrangement pattern of somatic ciliature (scheme). 24 – 25. Ventral and dorsal views of the same specimen. Double-arrowheads in Fig. 24 indicate SK<sub>1</sub> forming a curve at the end of cell while arrows mark the end of SK<sub>2</sub> on the ventral side. 26 – 27. Ventral views of two specimens, to show the different cell shapes, arrow marks the apical protrusion. 28. Dorsal view, to show the posterior end of SK<sub>1</sub> (double-arrowheads) and SK<sub>2</sub> (arrow). 29. To show the arrangement of extrusomes on dorsal side (arrows). 30. Right ventral view of cell, to demonstrate SK<sub>1</sub> and SK<sub>2</sub>. 31. Left dorsal view of cell showing SK<sub>1</sub> and SK<sub>2</sub>. 32. Right view of cell, showing SK<sub>1</sub> and SK<sub>2</sub>, posterior end of SK<sub>1</sub> extends across left side of cell and terminates at the left dorsal side. 33. Arrow marks the newly formed oral primordium which originates beneath SK<sub>2</sub>. 34. Macronucleus. Abbreviations. AM. Anterior membranelles. AP. Apical protrusion. Ma. Macronucleus. SK<sub>1</sub>. Somatic kinety 1; SK<sub>2</sub>. Somatic kinety 2. VM. Ventral membranelles. Scale bars: 21 – 27, 29 = 30  $\mu$ m; 28, 30 – 32 = 20  $\mu$ m, 33 – 34 = 10  $\mu$ m.

Somatic ciliature as shown in Figs 4 – 5, 8 – 10, 16, 19 – 20. Somatic cilia approximately 10  $\mu$ m long (Fig. 15, arrowheads), prominent in vivo, arranged as five somatic kineties. Five somatic kineties (SK<sub>1</sub>–5) always present, each composed of dikinetids, forming obliquely oriented parallel series on ventral side (Figs 4 – 5, 9 – 10, 20). Somatic kinety 1 originating on left side of cell and extending obliquely rightward across

ventral side toward anterior, turning back toward left and running transversely to dorsal side and curving toward posterior, terminating almost at posterior end of cell (Fig. 16, arrowhead). Somatic kinety 2 originating on left side of cell posterior to SK<sub>1</sub>, extending obliquely rightward and toward anterior across ventral side onto dorsal side, running back toward posterior and terminating almost at posterior



end of cell (Fig. 19). Dorsal portions of SK<sub>1</sub> and SK<sub>2</sub> with discontinuous segments (Figs 5, 10, 19). Somatic kineties 3 – 5 located in series posterior to SK<sub>2</sub>, originating on left side of cell, extending obliquely rightward and toward anterior across ventral side, ending at right edge of ventral side (Figs 8 – 10).

### 3.4 Systematic position of the new genus *Varistrombidium*

In our SSrRNA phylogenetic trees (Fig. 35), *Varistrombidium kielum* and *Omegastrombidium* associated as sister clade and supported by high PP (1.00) but variable bootstrap values (74 % ML, 61 % NJ, and 54 % MP). And this relationship was also supported in phylogenetic network (Fig. 36). As the trees revealed, they are invariably sister clade of other typical oligotrichs, that is, the assemblage of *Novistrombidium-Parallelostrombidium* but parallel to *Strombidium* species.

### 3.5 SSrRNA gene secondary structure of *Varistrombidium kielum* and its morphologically similar species

The predicted secondary structures agree with the generalized eukaryotic SSrRNA gene model (Neefs *et al.*, 1993).

Inspection of the sequence alignment shows some differences among oligotrichs in the variable region 2, including Helix 10, Helix E10-1, and Helix 11 (Figs 37 – 53). *Laboea* had only one loop in Helix 10 while all the other species had two which makes it markedly different from all the others (Fig. 53, double arrowheads). The nucleotides comprising Helix E 10-1 in all *Strombidium* species appears to form a linear helix with 3 interior loops, with the one near the base of the helix much larger than the other two (arrow in Figs 37 – 42), while *S. conicum* has only one interior loop and two small bulges which are composed of 1 – 3 nucleotides (Fig. 43, arrowheads). *Novistrombidium testaceum* has a longer Helix 11 compared with other species (Fig. 44). Three other *Novistrombidium* species, *N. sinicum* pop. 1, *N. sinicum* pop. 2, *N. orientale*, and its phylogenetically related genus *Parallelostrombidium* share the similar secondary structure in which Helix E10-1 has one interior loop and one small bulge composing of 3 nucleotides (Figs 45 – 48). The secondary structures of *Pseudotontonia* and *Spirotontonia* are similar except that the former has a longer Helix 10 and a small bulge (arrow in Fig. 51). The main difference between *Varistrombidium* and *Omegastrombidium* is that the former has three interior loops and one bulge in Helix E10-1, while the latter has one interior loop and two bulges (Figs 49 – 50).

### 3.6 Additional re-description of *Apostrombidium pseudokielum* Xu *et al.*, 2009

This species was recently reported in the monograph by Xu *et al.* (2009). However, the originally description lacks the statistic data, detailed

living morphology as well as some microphotographs (Xu *et al.*, 2009). Thus, we give some supplementary data (Table 1, and Figs 21 – 34).

**Table 1.** Measurements of attributes of *Varistrombidium kielum* (upper line) and *Apostrombidium pseudokielum* (lower line) performed on randomly selected sample of protargol-impregnated cells. Measurements in  $\mu\text{m}$ ; Max, maximum; Mean, arithmetic mean; Min, minimum; n, number of specimens; SD, standard deviation.

Characteristics	Min	Max	Mean	SD	n
Length of cell	53	74	65.2	6.6	15
	46	72	60.4	6.9	20
Width of cell	38	50	44.1	4.3	15
	34	50	39.6	4.4	20
Distance from apex	8	12	10.4	1.1	15
To cytostome	10	18	12.7	2.2	20
Length of macronucleus	15	20	18.3	2.0	15
	15	25	20.4	2.3	20
Width of macronucleus	9	15	10.2	1.8	15
	15	23	18.6	2.6	20
Number of anterior	15	17	16.0	0.8	15
Membranellae	16	18	17.0	0.7	20
Number of ventral	7	8	7.0	0.3	15
Membranellae	6	8	7.0	0.7	20

Living cells about  $50 - 75 \mu\text{m} \times 35 - 55 \mu\text{m}$ , slightly asymmetric cordiform (Figs 21, 26), posterior end of cell usually bluntly acuminate (Fig. 27). Apical protrusion conspicuous, about  $5 \mu\text{m}$  high (Figs 26 – 27, arrow). Buccal cavity not prominent, terminating approximately  $1/4 - 1/5$  of distance toward posterior end of cell (Fig. 21). Cytoplasm transparent, often filled with large ingested diatoms and small sized light reflecting particles (Figs 26 – 27). Extrusomes inconspicuous, ca.  $5 - 8 \mu\text{m}$  long, acicular in shape, arranged beneath shoulder area on the dorsal side of cell (Fig. 29, arrows). Single ovoid macronucleus with numerous small globular nucleoli ca.  $2 - 4 \mu\text{m}$  in size (Fig. 34). Micronucleus not detected.

Oral apparatus typical of genus. 16-18 anterior and 6 – 8 ventral membranellae. Bases of the ventral membranellae about  $3 - 6 \mu\text{m}$  in length, distinctly shorter than anterior ones, which are about  $8 \mu\text{m}$  long. Endoral membrane not detected. Girdle kinety discontinuous, divided into two parts, SK<sub>1</sub> and SK<sub>2</sub> as shown in Figs 23 – 25, 28, and 30 – 32. Both SK<sub>1</sub> and SK<sub>2</sub> starting from the end of cell on the dorsal side (Figs 25, 30), running across dorsal side of cell, reaching shoulder area, then extending along ventral side of cell and terminating at the cell end. Different from SK<sub>2</sub>, after reaching ventral end side of cell, SK<sub>1</sub> terminating at left caudal end (Figs 24, 32, arrows). Only one specimen in stomatogenesis was found in our

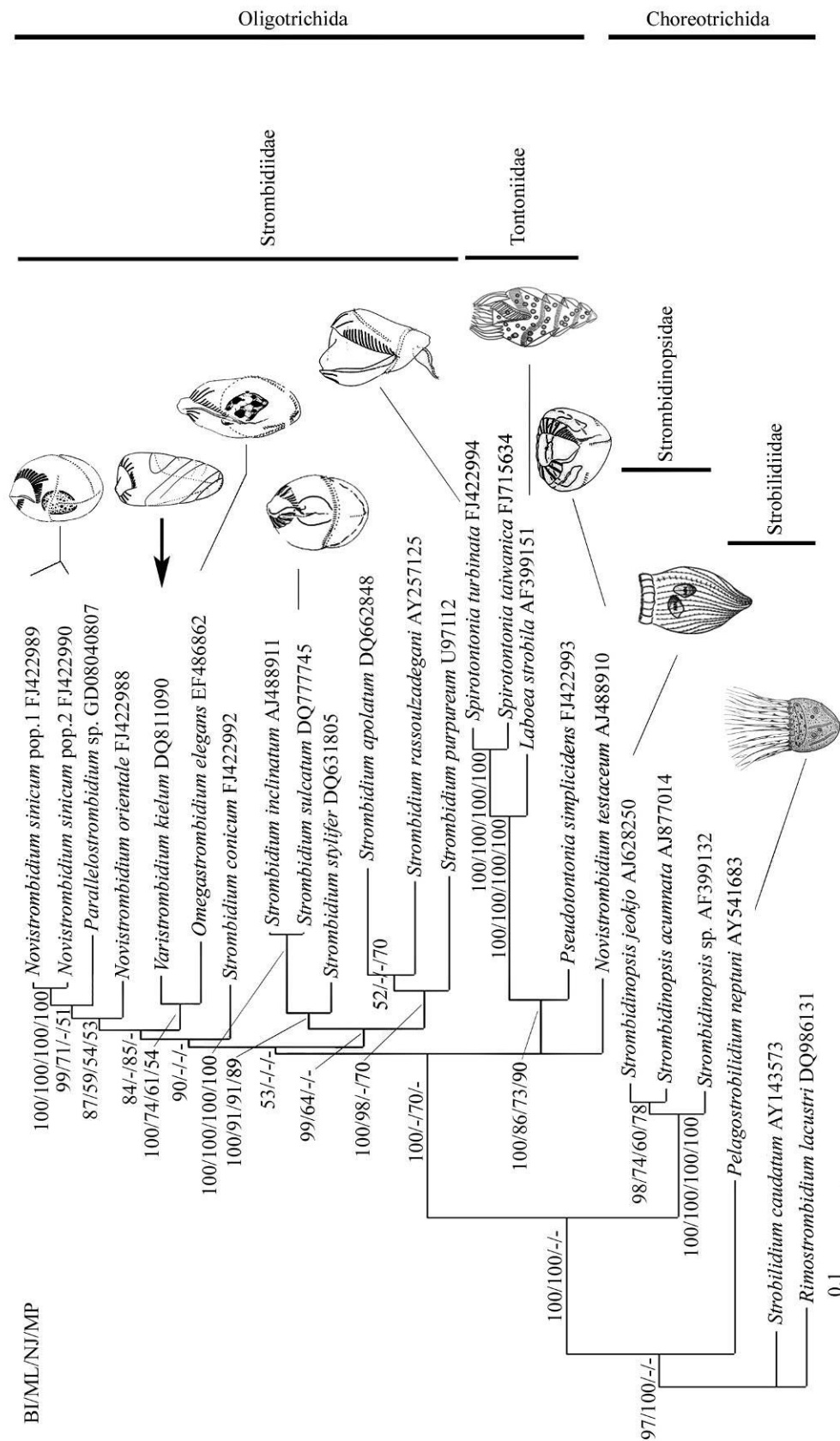


Fig. 35. Phylogenetic tree inferred from SSrRNA gene sequences. Numbers on branches are the following: Bayesian posterior probability value followed by bootstrap values from maximum likelihood (ML), Neighbor joining (NJ), and maximum parsimony (MP) analyses. Solid circles represent full bootstrap support in all three algorithms and hyphen ( - ) represents support values below 0.50/50 %.

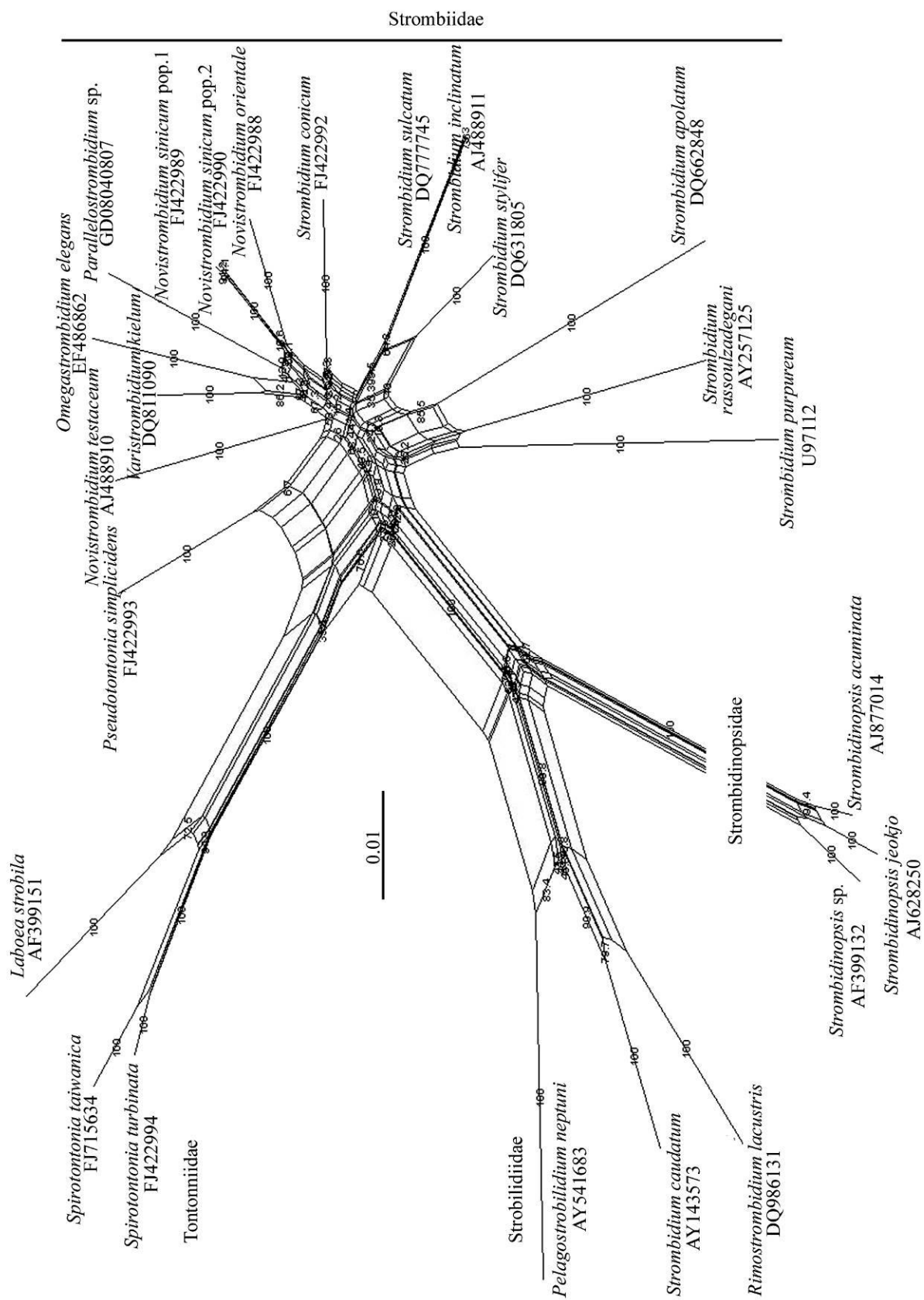
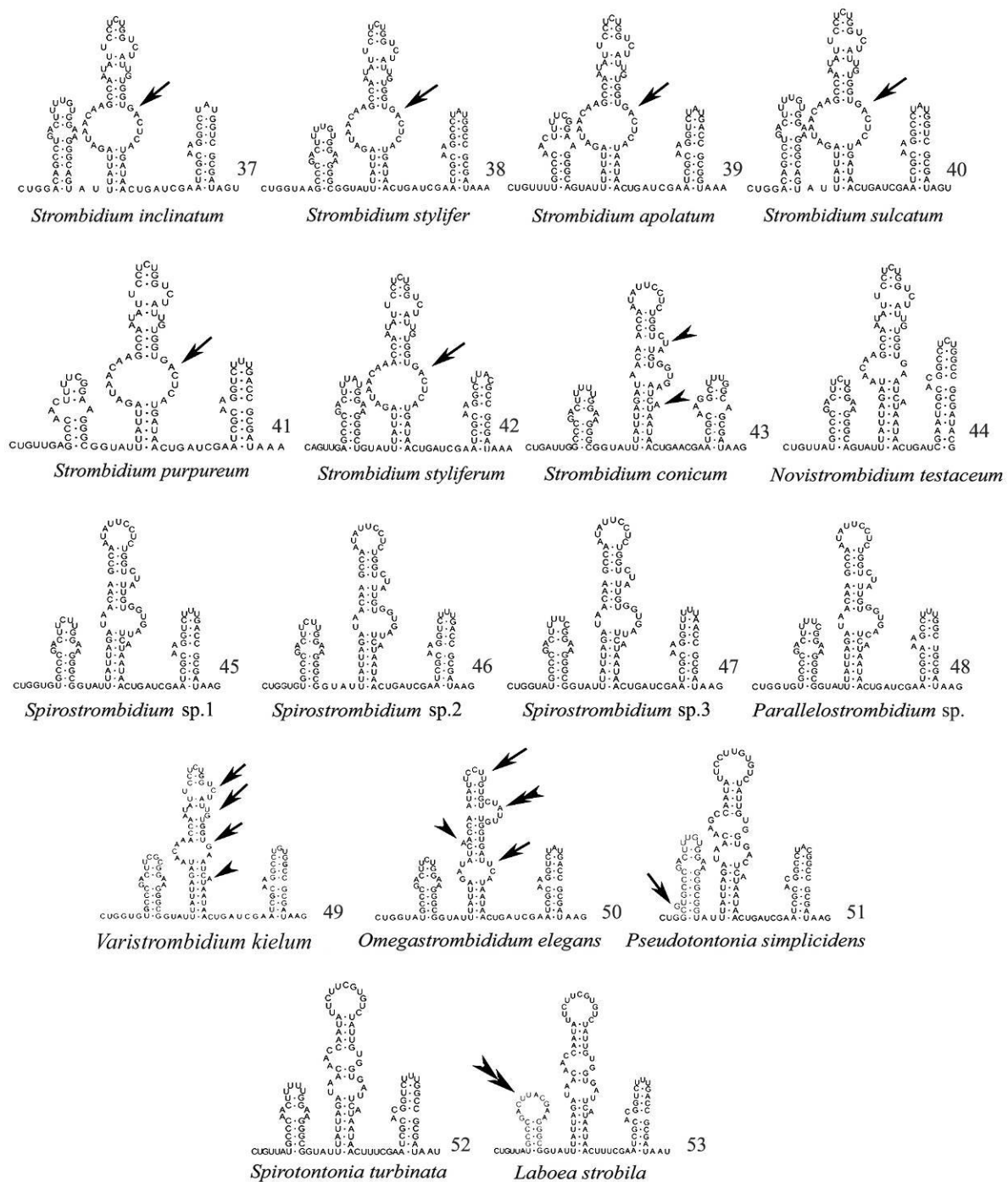


Fig. 36. Neighbor-Net reconstruction of the oligotrich ciliates as implemented in the program SplitsTree. The "core" Strombidiidae is marked in grey. The analysis is based on p-distances, the scale bar indicates 0.01 substitution/site.



Figs 37 – 53. Secondary structure of the small subunit rRNA molecule in the region of the Helices 10, E10-1, and 11 (from left to right) in 17 oligotrich ciliates.

protargol-stained slide, in which the new oral primordium originates from beneath SK<sub>2</sub> just below ventral membranelles (Fig. 33, arrow).

#### 4 Discussion

##### 4.1 Comparison of Qingdao population of *Varistrombidium kielum* with the type population and establishment of the new genus

Kahl (1932) described living cells of an oligotrich

collected from marine sand in Kiel Bay, Germany, and identified them as belonging to an unknown, possibly new species of *Strombidium* (Fig. 2). Much later, Maeda & Carey (1985) named this form *Strombidium kielum* in their revision of oligotrich ciliates, but information about its infraciliature has never been supplied. Consequently, we based identification of our organism on the basic characteristics of the living cells given accurately by Kahl (1932), namely size, slender



shape, general morphology of the buccal apparatus, and marine sand biotope. The population from Qingdao matches Kahl's (1932) description strongly with respect to these characteristics, and thus, we are confident that the two forms are conspecific.

Since the ciliary pattern of *Varistrombidium kielum* revealed by protargol staining is unique among known oligotrich ciliates, it is easy to be separated from other oligotrichs. The establishment of a new genus is thus verified.

#### 4.2 Systematic position of the new genus *Varistrombidium*

As revealed recently (Agatha, 2004), patterns of somatic ciliature are critical to the separation of oligotrich genera. All species of *Strombidium* have one somatic kinety oriented longitudinally on the ventral side and one kinety encircling the cell transversely to form a girdle (Xu *et al.*, 2009). By contrast, the ciliary pattern of *Varistrombidium* is quite unique in consisting of 5 spiralled somatic kineties, none of which are oriented like either kinety in the pattern typical of *Strombidium*. According to the evolution theory of oligotrichid ciliary patterns proposed by Agatha (2004), the oligotrichid kineties might originate from longitudinal ciliary rows then differentiate into different patterns. The ciliary pattern of the present form does not fit any patterns reported previously so it might be a transitional form and other transitional forms might have not yet been found since the ciliary pattern in *Omegastrombidium* differs greatly from that of *Varistrombidium*. *Omegastrombidium* has one girdle and one ventral kinety. The girdle kinety is horizontally orientated on dorsal side and kinety ends extend to posterior end of ventral side and one ventral kinety. While *Varistrombidium* has 5 spiralled somatic kineties and no ventral kinety. Further collection of molecular as well as morphological / stomatogenesis data might help to determine the phylogenetic position of *Varistrombidium*.

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## 寡毛类纤毛虫变游虫新属的建立及基尔变游虫(新属,新组合)的形态学重描述与系统地位分析

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**摘 要** 利用蛋白银染色技术对采自青岛沿海砂隙的寡毛类纤毛虫 *Strombidium kielum* 进行了形态学重描述,发现该种在寡毛类纤毛虫中具有独一无二的纤毛下器模式,因此为其建立了1新属 *Varistrombidium*,特征为具有5条斜穿虫体的体动基列,其中体动基列1和2延伸到虫体背部,终止于虫体尾端。对 *Varistrombidium kielum* (Maeda & Carey, 1985) nov. comb.

的小亚基 RNA 序列分析表明,该种位于 Strombidiidae 科内,与其形态学相近种 *Omegastrombidium elegans* 聚在一起。同时对其小亚基 RNA 序列可变区 2 的二级结构进行了预测并与其形态学相似种进行了比较。还对 *Apostrombidium pseudokielum* Xu *et al.*, 2009 进行了补充性描述。

**关键词** 小亚基 RNA, 二级结构, 黄海, 中国.

**中图分类号** Q959.11

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